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TITLE: Innate Anti-Breast Cancer Activity of -T cells: A Novel Biological and Clinical Approach to the Treatment of Relapsed or Refractory Breast Cancer

PRINCIPAL INVESTIGATOR: Richard D. Lopez, M.D.

CONTRACTING ORGANIZATION: University of Alabama at Birmingham
Birmingham, AL 35294

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14. ABSTRACT We initially identified a specific signaling pathway which inhibits apoptosis in human -T cells. We have exploited this pathway to develop the methodologies allowing the large-scale ex vivo expansion of viable apoptosis-resistant -T cells. Importantly, we have shown that apoptosis-resistant human -T cells retain significant innate (MHC-unrestricted) cytotoxicity against a wide variety of tumor cell lines, including human breast cancer cell lines. In this project, we have focused upon testing the hypothesis that -T cells – by virtue of their innate ability to recognize and kill epithelial-derived malignancies – play an important role in regulating the initial growth or spread of breast cancer in vivo and may also be of therapeutic utility. In this report, we summarize the findings we have made during the course of this project. In both the human pre-clinical work and in the mouse models, we have made the important discovery that -T cells are severely impaired in tumor-bearing hosts (human and mouse) compared to healthy controls – this possibly limiting our ability to use patient-derived (autologous) -T cells for therapy. However, data derived from animal studies clearly show that using -T cells derived from healthy donors (syngeneic or allogeneic) offers a feasible and rationale alternative approach when patient-derived (autologous) -T cells cannot be expanded for use clinically. This represents a significant conceptual advance and is the basis for new studies.					
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INTRODUCTION

We initially identified and characterized a CD2-mediated, interleukin (IL)-12-dependent signaling pathway which inhibits apoptosis in mitogen-stimulated human $\gamma\delta$ -T cells. We have since exploited this pathway to develop the methodologies allowing the large-scale ex vivo expansion of viable *apoptosis-resistant* $\gamma\delta$ -T cells – an undertaking until now, not possible. Importantly, we have shown that apoptosis-resistant human $\gamma\delta$ -T cells retain significant innate, major histocompatibility complex (MHC)-unrestricted cytotoxicity against a wide variety of human-derived tumor cell lines, including human breast cancer cell lines. Our efforts related to this proposal have remained focused upon testing the hypothesis that $\gamma\delta$ -T cells – by virtue of their innate ability to recognize and kill epithelial-derived malignancies – play an important role in regulating the initial growth or spread of breast cancer in vivo.

BODY

In this grant for which this report is generated (3 February, 2006 to 2 February, 2009) our accomplishments are presented in relation to the following tasks as outlined in the approved Statement of Work.

Task 1: To determine the extent to which $\gamma\delta$ -T cell numbers; $\gamma\delta$ -T cell innate antitumor capacity and $\gamma\delta$ -T cell expansion potential vary as a function of breast cancer clinical stage, clinical progression and clinical response to standard therapy.

FINDINGS:

1. Breast cancer patients appear to have a demonstrable numeric deficit of peripheral blood $\gamma\delta$ -T cells.

(Please note that at the time of this closing report, the statistical analysis reported here are only preliminary and are currently undergoing final analysis and will be presented in full upon publication of this and related work). Peripheral blood samples were obtained from breast cancer patients and healthy female controls. Flow cytometric analysis was performed as previously described. All cells were analyzed by FACS using a FACSDiva or a FACSCalibur flow cytometer (Becton Dickinson). A complete blood count along with a differential cell count was obtained using a Coulter analyzer. The percentage of cells staining for TCR $\gamma\delta$, TCRV δ 1 and TCRV δ 2 was determined within the lymphocyte gate as determined by FACS. The absolute number of $\gamma\delta$ T cells was calculated as the product of the absolute lymphocyte count and the percentage of cells staining for TCR $\gamma\delta$ by flow cytometry. Likewise, the subpopulations of V δ 1 and V δ 2 were calculated as the product of the absolute lymphocyte count and the percentage of cells staining for V δ 1 and V δ 2, respectively. Absolute lymphocyte count, CD3+, CD4+, and CD8+ T cell counts were not significantly different between patients and healthy controls. However, patients had substantially fewer $\gamma\delta$ -T cells/ μ l when compared to healthy controls. The significance or the biology of this finding is not yet known, but is addressed in the studies outlined in the mouse models described below.

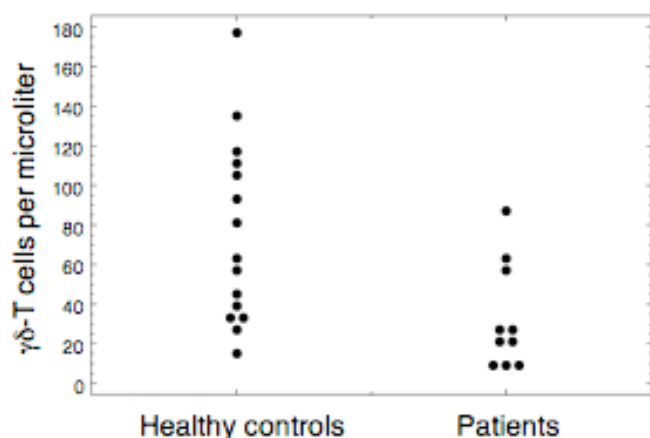


Figure 1. Comparison of peripheral blood $\gamma\delta$ -T cell counts between patients and healthy controls. $\gamma\delta$ -T cell counts obtained from patients (right) and aged matched, healthy controls (left) where each dot represents the $\gamma\delta$ -T cell count of one individual expressed as $\gamma\delta$ -T cells present per microliter ($\gamma\delta$ -T cells/ μ l) of blood. Absolute lymphocyte count, CD3+, CD4+, and CD8+ T cell counts were not significantly different between patients and healthy controls. The mean $\gamma\delta$ -T cell counts for patients was 33 $\gamma\delta$ -T cells/ μ l compared to that of healthy controls which was 75 $\gamma\delta$ -T cells/ μ l ($P=0.016$). Median $\gamma\delta$ -T cell counts for patients was 24 $\gamma\delta$ -T cells/ μ l (range, 7 to 147) compared to that of healthy controls which was 63 $\gamma\delta$ -T cells/ μ l (range, 16 to 175).

2. $\gamma\delta$ -T cells are difficult – if not impossible – to efficiently expand from peripheral blood obtained from patients with breast cancer.

Advances by ourselves and others have led to the development of both the scientific rationale as well as the technical means necessary to execute early-phase human clinical trials designed to exploit the innate antitumor properties of autologous (patient-derived) $\gamma\delta$ -T cells. Though it remains to be determined how this might best be accomplished, two general approaches are currently being taken in this regard. One approach being pursued by others relies upon the use of pharmacologic compounds to activate $\gamma\delta$ -T cells *in vivo*. The alternative approach (which we favor) relies upon the *ex vivo* expansion (and subsequent re-infusion) of peripheral blood-derived $\gamma\delta$ -T cells. Importantly, findings from our studies (above) now suggest that: (1) when compared to healthy donors, endogenous $\gamma\delta$ -T cells appear to be decreased in numbers in the peripheral blood of breast cancer patients, and (2) only in a proportion of patients with breast cancer is it possible to efficiently expand endogenous $\gamma\delta$ -T cells *ex vivo*. This is shown in **Figure 2** where $\gamma\delta$ -T cells are *ex vivo* expanded from blood obtained from healthy donors and patients.

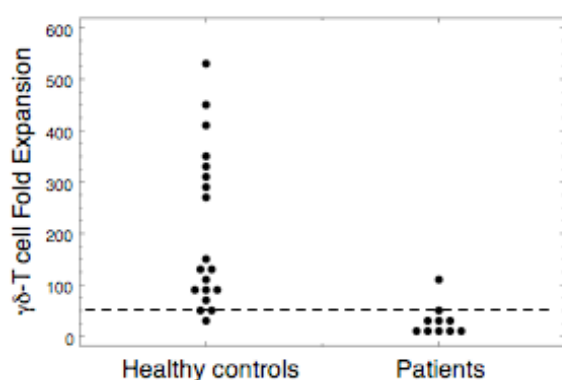


Figure 2. Comparison of $\gamma\delta$ -T cell expansion between patients and healthy controls. The expansion of $\gamma\delta$ -T cells was undertaken as we have previously described. On day 0 and day 15 of culture, peripheral blood mononuclear cells (PBMC) were stained using fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC)-directly conjugated mAbs recognizing CD3, CD5, TCR- $\gamma\delta$ or TCR- $\alpha\beta$. Cells were immediately analyzed using a FACScalibur flow cytometer (Becton Dickinson). The percentage of viable T cells within the lymphocyte gate was determined allowing for the determination of the absolute number of $\gamma\delta$ -T cells in culture. Fold expansion was determined by dividing the absolute number of $\gamma\delta$ -T cells present on day 15 by the number of $\gamma\delta$ -T cells present at initiation of cultures. The mean $\gamma\delta$ -T cell expansion for patients was 29 fold compared to that of healthy controls which was 209 fold ($P=0.008$). Median $\gamma\delta$ -T cell expansion for patients was 22 fold (range, 1 to 118 fold) compared to that of healthy controls which was 137 fold (range, 33 to 534 fold).

Indeed, as recently presented at the 3rd International $\gamma\delta$ -T cell Conference (Marseille, France, May 21-23, 2008), a number of investigators – including ourselves – now report that regardless of the means used to activate and/or expand $\gamma\delta$ -T cells, in very few tumor-bearing individuals, $\gamma\delta$ -T cells expand poorly *ex vivo*, irrespective of the histological subtype of the underlying cancer. Accordingly, by consensus, patients are now classified as "non-responders" if $\gamma\delta$ -T cells expand *ex vivo* by less than 50-fold.

3. *Ex vivo* expanded $\gamma\delta$ -T cells derived from patients with metastatic breast cancer (actively undergoing therapy) retain cytolytic activity against human breast cancer cells *in vitro*.

Despite the high likelihood that $\gamma\delta$ -T cells cannot be reliably expanded from peripheral blood of patients (in contrast to healthy donors), data shown in **Figure 3** are provided to demonstrate that when $\gamma\delta$ -T cells can indeed be expanded from a patient, these cells nevertheless retain innate antitumor activity against human breast cancer cells *in vitro*. For these studies, $\gamma\delta$ -T cells were isolated from cultures derived from breast cancer patients who were actively undergoing therapy suggesting that future clinical trials (with appropriate screening) are indeed feasible.

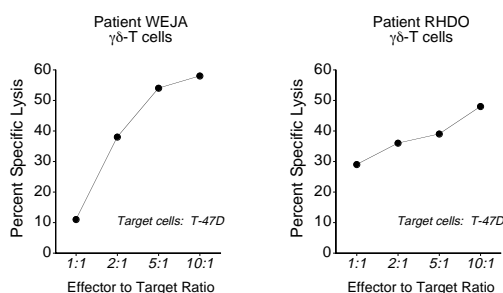


Figure 3. $\gamma\delta$ -T cells from two separate patients with metastatic breast cancer display cytotoxic activity against human breast cancer cell line T-47D. $\gamma\delta$ -T cells were first expanded (described above) then purified from peripheral blood mononuclear cell cultures derived from patient WEJA (left) and patient RHDO (right). Purified $\gamma\delta$ -T cells were then assessed for their *in vitro* antitumor activity measured against human breast cancer cell line T-47D as previously described. Data are presented as percent specific lysis of T-47D target cells after 4 hr co-culture with the corresponding effector $\gamma\delta$ -T cells. Effector to target ratios ranged from 1:1 to 10:1.

BIOLOGICAL AND PRACTICAL ISSUES ENCOUNTERED, AND THEIR SOLUTIONS***Biological Issues Encountered.***

The emerging view is that there exists a poorly-defined cancer-associated impairment of $\gamma\delta$ -T cells in some patients which contributes to the observed poor *ex vivo* $\gamma\delta$ -T cell expansion. Nevertheless, it is evident that $\gamma\delta$ -T cells present in certain selected patients still remain capable of being activated and/or expanded (i.e., >50-fold expansion). There are two possible solutions to this problem.

The first solution relies upon learning how to prospectively identify patients in whom $\gamma\delta$ -T cells will expand *ex vivo* – and thus who could be included in clinical trials. Toward this end, our findings reported here have provided the rationale for key new studies which are now ongoing as part of a recently funded Breast Cancer SPORE Grant (R. Lopez, Project 4 co-leader). These studies focus on identifying the factors which might predict for adequate (or poor) $\gamma\delta$ -T cell expansion. This includes examination of $\gamma\delta$ -T cell activation markers, cytokine production profile, etc., as well as defining the relative percentage of other key cells in peripheral blood (i.e., CD4+CD25+ T_{reg} cells) which might impair $\gamma\delta$ -T cell activation/expansion *ex vivo*.

The second solution is to develop an alternative source of $\gamma\delta$ -T cells. As it appears possible to expand $\gamma\delta$ -T cell from the peripheral blood of healthy donors (and from some patients with breast cancer), the inescapable conclusion which we draw from the **Figure 1** and **Figure 2** is that on account of diminished numbers and/or poor expansion capacity, the use of patient-derived (i.e., **autologous**) $\gamma\delta$ -T cells for cancer immunotherapy may be undesirable or even impossible. With this in mind, in our slightly modified animal studies, we initiated studies to address the issue of using adoptively transferred **allogeneic** $\gamma\delta$ -T cells (derived from healthy donors) for the treatment of advanced or recurrent breast cancer.

Practical Issues Encountered.

A key goal of our pre-clinical work was to refine our novel *ex vivo* expansion methods to allow for the large-scale production of a cell therapy product which could be administered clinically. However, transition to clinical-scale production of $\gamma\delta$ -T cells has been curtailed by the loss of the clinical-grade (cGMP) anti-CD2 clone 6G4 previously obtained from Baxter Healthcare as Baxter informed us that they would no longer produce the cGMP grade anti-CD2 antibody used in our $\gamma\delta$ -T cell manufacturing process. It is important to note that low patient accrual for our studies outlined in Aim 1 is directly related to our decision not to perform expansion and functional studies on patient-derived samples using less than cGMP grade materials – as these studies would not be relevant to future clinical studies if we used reagents which we could never use clinically.

Possible solutions to the problem (not directly pertinent to this grant, but to studies which are now ongoing and which arose directly from the accomplishments of these current studies).

- A. *New sources for anti-CD2 antibody.* Two approaches are being taken to secure a clinical grade anti-CD2 antibody. (1) Our first option was to attempt to obtain cGMP grade anti-CD2 antibody (clone S5.2) from BD Biosciences. BD has agreed in direct discussions with us to move forward with the manufacturing of near-GMP antibody adequate for FDA approval of a Phase I trial. BD has produced a test lot for our evaluation. As a practical matter, BD currently manufactures the S5.2 antibody in a cGMP *in vitro* diagnostics grade (IVD) and will alter the manufacturing process to exclude sodium azide. (2) Alternatively, we will obtain the 6G4 antibody from Baxter's supplier Sanquin Pharmaceutical Services. Using the identical procedures, Sanquin would assume the manufacturing of clinical grade antibody. Cost would be somewhat reduced by the fact that manufacturing and validation SOPs are in place and the master clone bank has been certified free of adventitious virus.
- B. *Adopt the use of an alternative means to expand human $\gamma\delta$ -T cells.* As described above, other methods now exist to *ex vivo* expand human $\gamma\delta$ -T cells, including methods employing the use of synthetic phosphoantigens (such as BrHPP) or bisphosphonates (such as zoledronic acid, Zometa®). However, unlike our methods, these other methods do not provide protection from activation-induced cell death (apoptosis). Nevertheless, if we are unable to secure clinical grade anti-CD2 antibodies in a timely manner, we have made contingent plans to proceed with our clinical trial using compounds such as BrHPP or Zometa® for use in the generation of $\gamma\delta$ -T cells. We have recently received (free of

charge) a batch of cGMP manufactured BrHPP (Phosphostim) from *Innate Pharma*, Marseille, France. We have completed early optimization studies using BrHPP in direct comparison with our methods (which we must perform using non-cGMP grade antibody). We will also combine the two methods as we believe that aspects of our methods impart resistance to apoptosis in $\gamma\delta$ -T cells, and in conjunction with BrHPP, may yield a more potent (i.e., apoptosis-resistant) effector population of $\gamma\delta$ -T cells. Similar studies are planned using Zometa[®], but we have since shown that Zometa[®] is far less potent than BrHPP or our methods when used to expand $\gamma\delta$ -T cells.

Task 2: To further refine our understanding of the in vitro biology (recognition and effector functions) of the antitumor cytotoxicity mediated by human $\gamma\delta$ -T cells against human breast cancer cells.

FINDINGS:

Given the loss of access to the reagents required for these studies explicitly outlined in this section, we have been unable to complete these studies in the detail originally proposed. Nevertheless, studies we have been able to perform indeed confirm that $\gamma\delta$ -T cells expanded from patients with breast cancer do indeed kill tumor cells in a T-cell receptor-dependant manner, similar to $\gamma\delta$ -T cells obtained from healthy donors, as we have previously published (1). Similarly, we have also confirmed that killing of tumor cells by $\gamma\delta$ -T cells derived from patients occurs through the perforin/granzyme pathway, similar to $\gamma\delta$ -T cells obtained from healthy donors, as we have previously published (1). We have since determined (negative finding) there are no gross differences observed in the expression of key activation markers, adhesion molecules as well as cytokine production when comparing $\gamma\delta$ -T cells obtained from healthy donors and those obtained from patients with breast cancer.

Task 3: Pre-clinical models for the adoptive cellular immunotherapy of breast cancer. To determine the extent to which $\gamma\delta$ -T cells can regulate the growth and metastasis of breast cancer cells in vivo using pre-clinical animal models.

FINDINGS:

Treatment of tumor-bearing animals with human $\gamma\delta$ -T cells (xenograft model)

As we initially showed in our grant proposal, human breast cancer tumors could be controlled by intravenously delivering human $\gamma\delta$ -T cells into tumor-bearing mice. However, as our proposed studies using the human tumor xenograft model required the ex vivo expansion of human $\gamma\delta$ -T cells *using clinical-grade reagents to which we lost access*, we have been unable to complete these studies to our satisfaction – owing to poor cell expansion, poor viability and unreliable antitumor activity obtained when using non-clinical grade reagents to grow human $\gamma\delta$ -T cells. Nevertheless, we provide a brief summary of our relevant human xenograft studies – and importantly, link these studies to our subsequent successful studies performed in our syngeneic mouse model.

In vivo bio-distribution. Human breast cancer tumors were established in nude mice. Animals harboring human tumors then received radio-labeled human $\gamma\delta$ -T cells by intravenous (i.v.) injection. Animals were subsequently assessed to determine $\gamma\delta$ -T cell kinetics, biodistribution, and to the extent possible, tumor responses. In these studies, two related experiments (n=15 mice/experiment) were conducted. The first experiment used normal nude mice without tumors, while the second experiment used nude mice bearing palpable (4 mm) Luc-positive 2LMP breast tumors (a luciferase positive subclone of MDA-MB-231) implanted in the mammary fat pad. The fate of radio-labeled $\gamma\delta$ -T cells after i.v. injection was assessed by gamma camera imaging and SPECT/CT in both experiments. Detailed analyses was performed at 3 time points for each of the two experiments (6 hr, 24 hr, 72 hr; n=5 mice/time point). As shown in A) planar images and B) SPECT/CT fused images of tumor-bearing mice treated with radio-labeled human $\gamma\delta$ -T cells were obtained. In these studies, $\gamma\delta$ -T cells were labeled Indium-111. Using gamma camera imaging and SPECT/CT, we were unable to localize radio-labeled $\gamma\delta$ -T cells into established tumors at 6 hr, 24 hr, 72 hrs. We attribute this either to the inability of human $\gamma\delta$ -T cells to track/home appropriately in the mouse. Alternatively, this could be attributed to the use of non-clinical grade reagents to grow human $\gamma\delta$ -T cells, thus resulting in suboptimal cell viability or antitumor activity.

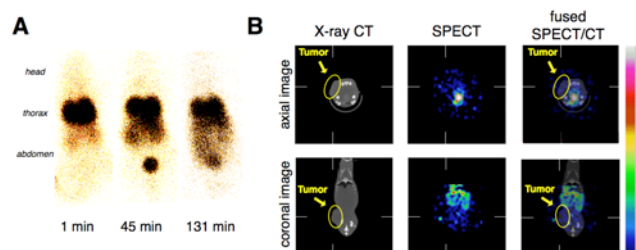


Figure 4. The fate of ^{111}In -labeled $\gamma\delta$ -T cells after i.v. injection: SPECT/CT Imaging: Radiotracer images were obtained using the small animal SPECT/CT system (X-SPECT, GammaMedica, Inc.) which was configured to detect ^{111}In . **A)** planar images performed using an Anger 420/550 Mobile Radioisotope Gamma camera (Technicare, Solon, OH) equipped with a pinhole collimator. **B)** Non-invasive images were generated utilizing the rotating dual gamma cameras of the SPECT device. Near simultaneously, X-ray CT images (10x10 cm field of view; with a 50 μm resolution) were also obtained. Using software provided with the SPECT/CT system, fused images of radiolabeled cells and mice were generated at a spatial resolution of ~ 1 mm. Fused images were then represented as 1 mm thick tomographic slices, or as volume renderings allowing for the precise anatomical localization of human $\gamma\delta$ -T cells within whole living animals.

Mouse syngeneic breast cancer model

Tumorigenic mouse breast cancer cells line 4T1 (derived from BALB/c) were used to establish disease in syngeneic BALB/c animals. Studies were then performed to assess the immunotherapeutic potential of adoptively transferred $\gamma\delta$ -T cells in the setting of established disease. $\gamma\delta$ -T cells used for these adoptive transfer studies were obtained from spleen cells derived from BALB/c mice lacking $\alpha\beta$ -T cells. Spleen mononuclear cells were isolated by density gradient centrifugation (800 \times g, 15 min) using Ficoll-Paque Plus (Amersham). Cells were cultured in a manner similar to methods developed for expanding human $\gamma\delta$ -T cells (2), but employing mouse cytokines and reagents as indicated (3). Spleen cell cultures were initiated at a density of 5×10^6 cells per ml in RPMI-1640 with 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 50 μM 2-ME. On the day of culture initiation (day 0), cells were transferred to tissue culture wells first coated with rat anti-mouse CD2 mAb clone RM2-5 (BD Bioscience). Recombinant mouse interferon- γ (1,000 U/ml, R&D Systems) and recombinant mouse IL-12 (10 U/ml, R&D Systems) were then added. After 24 hours (day 1), 3 volumes of fresh culture media was added. Cultures were then stimulated with 10 ng/ml anti-CD3 mAb clone 145-2C11 (BD Bioscience) and 300 U/ml mouse recombinant IL-2, (R&D Systems). Fresh medium with 10 U/ml human IL-2 (Roche Diagnostics) was added every 3 days. At day 8, cells were harvested. Purity of $\gamma\delta$ -T cells was assessed determined using a FACS Calibur flow cytometer (Becton Dickinson) employing directly conjugated hamster anti-mouse antibodies (CD3-APC, 145-2C11; TCR $\gamma\delta$ -FITC, GL3; BD Bioscience). Cell viability was determined by FACS or by fluorescent microscopy as described (2).

Significant results and their interpretation:

- 1. Syngeneic (BALB/c)-derived mouse $\gamma\delta$ -T cells kill BALB/c-derived 4T1 cells in vitro, but not non-malignant BALB/c-derived cells.** Key *in vitro* data firmly establishes that syngeneic BALB/c-derived $\gamma\delta$ -T cells can indeed kill syngeneic mouse 4T1 cells. Effector $\gamma\delta$ -T cells (generated as described above) were co-cultured with target cell lines 4T1 (tumor cells) and BALB/3T3 (non-malignant BALB/c fibroblast cell line) at effector to target (E:T) ratios of 1:1, 5:1, and 10:1 in a standard cytolytic assay. Specific cytotoxicity was determined and was expressed as percent specific lysis. Routinely, 4T1 (tumor cells) but not BALB/3T3 cells were killed at all E:T ratios. These studies are particularly important in the context of the subsequent in vivo immunotherapy models.
- 2. In vivo anti-tumor effects.** Mouse mammary tumor cell lines (4T1) were established in the mammary fat pad of healthy BALB/c mice. Animals harboring tumors were then treated with ex vivo expanded syngeneic BALB/c-derived $\gamma\delta$ -T cells delivered by intravenous injection. Tumor mass are being assessed after therapy using physical inspection. As described (4), tumor measurements are converted to a calculated tumor weight using the formula: Calculated Tumor Weight (mg) = [Width (mm)² \times Length (mm)] / 2. Assessment of pulmonary metastases in treated and untreated mice also being performed, as described (5) with the following modifications. At necropsy, lungs from each animal have been removed en bloc, weighed then fixed in formalin for subsequent analysis. Formalin-fixed lungs will be sectioned then embedded in paraffin for H&E staining. Digital images of lung sections will be produced using a Nikon Coolscan V ED scanner employing Nikon Scan 4.0 software. Morphometric analysis of lung

section photomicrographs will be performed using ImageJ software (version 1.37v, W. Rasband, NIH, USA, <http://rsb.info.nih.gov/ij/>). Tumor involvement of lung sections will be determined by expressing the area of lung parenchyma involved with tumor (dark blue) as a percentage of total lung parenchyma. **Please note that at the time of this final report, data is actively undergoing analysis as these studies were just recently completed.** These findings will be presented in full detail when these data are published. Preliminary analysis clearly indicates however, that substantially smaller primary tumors, as well as fewer metastatic lesions are seen in treated versus untreated mice. These studies support the view that it our model of $\gamma\delta$ -T cell-based immunotherapy is biologically feasible.

- 3. Localization of adoptively-transferred syngeneic $\gamma\delta$ -T cells to tumor sites: The fate of ^{111}In -labeled $\gamma\delta$ -T cells after adoptive transfer.** Studies in **Figure 5** show A) planar images and B) SPECT/CT fused images of tumor-bearing mice treated with radio-labeled syngeneic mouse $\gamma\delta$ -T cells. In these studies, $\gamma\delta$ -T cells were labeled using In-111, where 20 million radiolabeled mouse $\gamma\delta$ -T cells were injected intravenously into BALB/c mice bearing syngeneic BALB/c-derived 4T1 mammary tumor cells. Using gamma camera imaging and SPECT/CT, radio-labeled $\gamma\delta$ -T cells were clearly seen localizing into established tumors at both 24 hr and 72 hrs (n=5 mice/time point). These studies are currently being correlated to the anti-tumor effects of $\gamma\delta$ -T cells as established in the studies noted above.

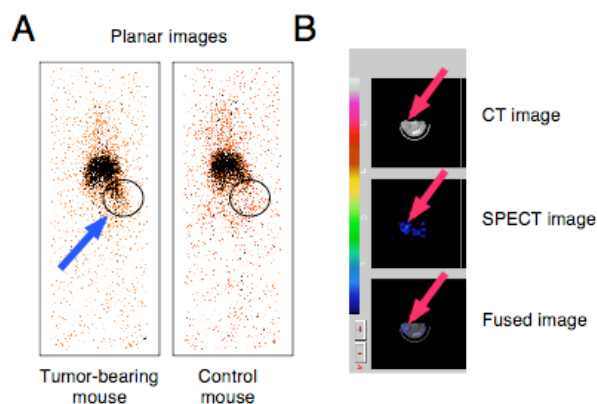


Figure 5. SPECT/CT Imaging: Eight-week old female BALB/c mice were sham-injected with saline, or received 4×10^4 4T1 mammary adenocarcinoma cells in the mammary fat pad. After 20 days, mice were injected intravenously with 5×10^6 BALB/c-derived ^{111}In -labeled $\gamma\delta$ T cells. A) planar images performed using an Anger 420/550 Mobile Radioisotope Gamma camera (Technicare, Solon, OH) equipped with a pinhole collimator. Animals were imaged at 6, 24, and 48 hours after intravenous injection with $\gamma\delta$ -T cells. B) Images were obtained using the small animal SPECT/CT system (X-SPECT, GammaMedica, Inc.) which was configured to detect ^{111}In . Non-invasive images were generated utilizing the rotating dual gamma cameras of the SPECT device. Near simultaneously, X-ray CT images (10x10 cm field of view; with a 50 μm resolution) were also obtained. Using software provided with the SPECT/CT system, fused images of radiolabeled cells and mice were generated at a spatial resolution of ~ 1 mm. Fused images were then represented as 1 mm thick tomographic slices, or as volume renderings allowing for the precise anatomical localization of $\gamma\delta$ -T cells within whole living animals.

- 4. Bio-distribution of radio-labeled $\gamma\delta$ -T cells.** In these studies, (**Figure 6**) animals treated with radiolabeled $\gamma\delta$ -T cells were sacrificed at 48 hours. The indicated tissues were surgically removed and radioactivity was quantified using a gamma scintillation counter. Results of these studies are expressed as a percent of injected dose of $\gamma\delta$ -T cells localizing to a specified target tissue (injected dose/gram of tissue). Two very remarkable findings are noted. First, these studies demonstrate for the first time that $\gamma\delta$ -T cells can indeed localize to a mammary tumor. This has never before been shown. Importantly, when compared to several other tissues, the percentage of the total dose of $\gamma\delta$ -T cells localizing to the tumor was substantial. While not specifically evaluated in this study, we speculate that the $\gamma\delta$ -T cells which localize to tumors are quite likely exerting anti-tumor effects. Studies to confirm this are currently underway in our laboratory. Second, it is evident that significantly fewer $\gamma\delta$ -T cells localize to the spleen of tumor-bearing mice when compared to the localization occurring in healthy control mice. We hypothesize that in tumor-bearing mice, $\gamma\delta$ -T cells display alternate trafficking due to their interactions with tumor cells. More specifically, it is well known that 4T1 can spontaneously metastasize when established in the anatomically correct site (i.e., mammary fat pad), as we have performed in these studies. This line of reasoning leads us to believe that the $\gamma\delta$ -T cells have localized both to the primary tumor as well as to sites of disseminated disease, which may account for the observed diminished localization to the spleen. Alternatively, and consistent with our model, we propose that $\gamma\delta$ -T cells are actively undergoing apoptosis in tumor bearing mice, resulting in the failure of these cells to localize to the spleen – since dead cells and their associated radioactive label will not likely localize to any specific tissue.

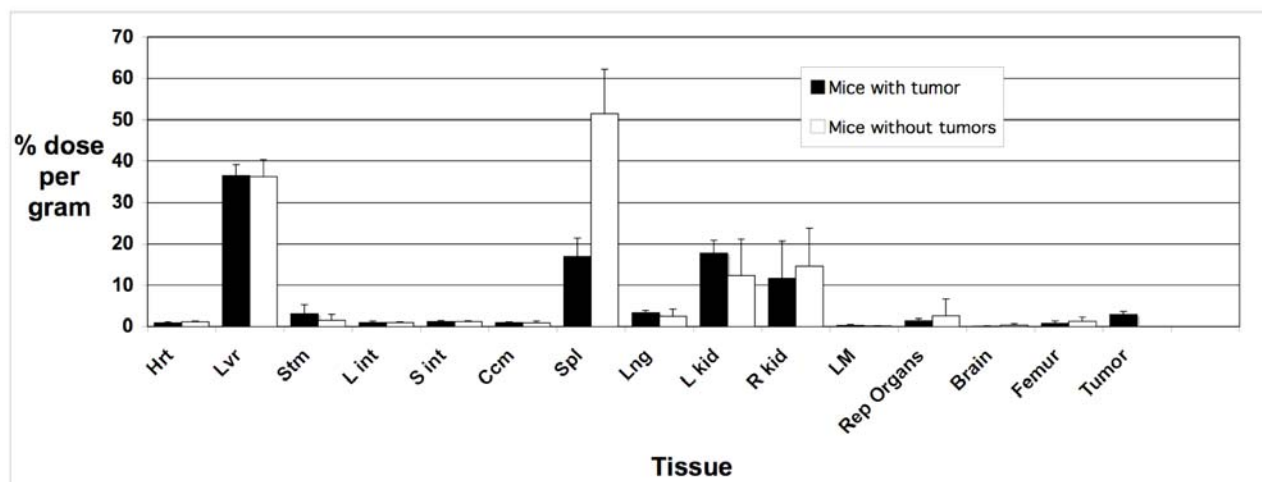


Figure 6. Bio-distribution of delivered $\gamma\delta$ -T cell dose expressed as a percent of dose per gram of tissue of interest. In these studies, healthy mice (open bars) or tumor-bearing mice (solid bars) were injected with radio-labeled syngeneic mouse $\gamma\delta$ -T cells. All animals were sacrificed 48 hours later and tissues were collected then subjected to gamma scintillation counting with results expressed as the percent of injected dose per gram of target tissue. Tissues are as follows: heart (Hrt); liver (Lvr); stomach (Stm); large intestine (L int); small intestine (S int); cecum (Ccm) and tumor. Results are expressed as the mean \pm STD. Results are corrected for counts found in the blood to exclude the effect of pooling of blood in tissues.

Indeed, on account of the work performed in this grant, we now hypothesize that in the majority of tumor-bearing hosts (mice or humans), the $\gamma\delta$ -T cell compartment is irreversibly "damaged" or "exhausted" – this possibly occurring in a tumor-dependent manner, quite possibly the result of tumor cells inducing apoptosis in tumor-reactive $\gamma\delta$ -T cells. This is supported in part by our current pre-clinical findings, as well as key findings made by others in different disease models (6). This is also partially supported by data where we compare the absolute $\gamma\delta$ -T cell count per mm^3 of blood between wild type healthy (control) mice and otherwise healthy mice which harbor 4T1 cells injected into the fat pad. In comparing healthy mice ($N=15$) with tumor-bearing mice ($N=11$), the respective $\gamma\delta$ -T cell counts (cells/mm^3) were 50 ± 31 $\gamma\delta$ -T cells/ mm^3 and 36 ± 15 $\gamma\delta$ -T cells/ mm^3 . While statistical analysis reveals that this is still only a very strong trend ($P>0.05$), additional studies are underway using larger numbers of mice to strengthen this point. Importantly, when $\alpha\beta$ -T cell counts from the same animals were determined, no differences were seen between healthy mice (4165 ± 1320 $\alpha\beta$ -T cells/ mm^3) and tumor bearing mice (3848 ± 1709 $\alpha\beta$ -T cells/ mm^3).

KEY RESEARCH ACCOMPLISHMENTS

- 1. We have established that breast cancer patients appear to have a demonstrable numeric deficit of peripheral blood $\gamma\delta$ -T cells.** Accumulating data suggests strongly that when compared to healthy donors, endogenous $\gamma\delta$ -T cells appear to be decreased in numbers in the peripheral blood of breast cancer patients – a finding which is consistent with the findings we and others have made in other disease models. This has never before been reported in breast cancer. Whereas the biological significance or the root cause of this $\gamma\delta$ -T cell deficit is not known, this finding has major implications in the design of future clinical trials intended to use patient-derived (autologous) $\gamma\delta$ -T cells for immunotherapy.
- 2. We have established that $\gamma\delta$ -T cells are difficult – if not impossible – to efficiently expand from peripheral blood obtained from patients with breast cancer.** Although the ability to expand $\gamma\delta$ -T cells from peripheral blood derived from patients with breast cancer is diminished, it is important to note that in a small proportion of these patients it is possible to efficiently expand endogenous $\gamma\delta$ -T cells *ex vivo*.
- 3. We have established that *ex vivo* expanded $\gamma\delta$ -T cells derived from patients with metastatic breast cancer (actively undergoing therapy) retain cytolytic activity against human breast cancer cells *in vitro*.** As our findings suggest that $\gamma\delta$ -T cells can be expanded from patients with metastatic

breast cancer who are actively undergoing therapy, we conclude that future clinical trials (with appropriate screening) are still feasible in selected patients.

4. **We have established that the treatment of tumor-bearing animals with human $\gamma\delta$ -T cells (xenograft model) is not an adequate biological or even pre-clinical model to assess anti-tumor efficacy given our inability to localize human $\gamma\delta$ -T cells to xenografted human tumor cells.** We attribute these experimental failures to either the inability of human $\gamma\delta$ -T cells to track/home appropriately in the mouse, or alternatively, this could be attributed to the use of non-clinical grade reagents to grow human $\gamma\delta$ -T cells, thus resulting in suboptimal cell viability and in vivo function.
5. **We have established that syngeneic (BALB/c)-derived mouse $\gamma\delta$ -T cells kill BALB/c-derived 4T1 cells in vitro, but not non-malignant BALB/c-derived cells.** These studies are particularly important in the context of the subsequent in vivo immunotherapy models – especially those involving human trials in which patient-derived (autologous) $\gamma\delta$ -T cells will be used for immunotherapy.
6. **We have established a model to assess the in vivo anti-tumor effects of syngeneic mouse $\gamma\delta$ -T cells against syngeneic mouse mammary tumors.** This model relied upon the acquisition of a BALB/c mouse lacking $\alpha\beta$ -T cells (which we acquired). Using this mouse as a source of pure $\gamma\delta$ -T cells has never before been attempted. Although early and not conclusive, our early findings establish that substantially smaller primary tumors, as well fewer metastatic lesions are seen in treated versus untreated mice. These studies support the view that it our model of $\gamma\delta$ -T cell-based immunotherapy is biologically feasible.
7. **We have established that adoptively-transferred syngeneic $\gamma\delta$ -T cells localize to sites of tumor.** These studies are currently being correlated to the anti-tumor effects of $\gamma\delta$ -T cells as established in the studies noted above.

REPORTABLE OUTCOMES

Manuscripts

- None currently. Work described here however is in the process of being prepared for publication.

Abstracts, presentations

- Beck BH, Liu Z and **Lopez RD**. Biological rationale for the adoptive transfer of allogeneic $\gamma\delta$ -T cells for the immunotherapy of malignancies of hematolymphoid or epithelial origin. 3rd International Conference on $\gamma\delta$ -T cells. May 21, 2008, Marseille, France.
- Bridges M, Lamb LS and **Lopez RD**. Antitumor activity of $\gamma\delta$ -T cells expanded from peripheral blood obtained from patients with metastatic breast cancer who are actively undergoing therapy. Department of Defense Breast Cancer Research Program (BCRP) Era of Hope Meeting, June 2008, Baltimore MD.

Animal models

- We have established a new animal model to assess the in vivo anti-tumor effects of syngeneic mouse $\gamma\delta$ -T cells against syngeneic mouse mammary tumors. This model relied upon the acquisition of a BALB/c mouse lacking $\alpha\beta$ -T cells (which we acquired). Using this mouse as a source of pure $\gamma\delta$ -T cells has never before been attempted, and thus represents an important practical and conceptual advance.

Funding applied for based on work supported by this award

- **Funded**

Title: UAB Breast Cancer SPORE Project 4: "Gamma/Delta T cell Immunotherapy of Breast Cancer"
Agency: NCI
Type: SPORE Grant (P50-CA08019)
Years: 2007-2012

SPORE P.I. Kirby I. Bland, M.D.

Project 4 co-directors: **LOPEZ, RD** and Zinn, Kurt.

Amount: \$210,651 per year, direct costs for Project 4.

Project Goals: Project 4 is a series of pre-clinical *in vitro* laboratory and *in vivo* animal studies of human $\gamma\delta$ -T cell anti-breast cancer activity. The project includes a phase-I clinical trial for the delivery of autologous $\gamma\delta$ -T cells to patients with relapsed breast cancer.

- ***Submitted and under review***

Title: Adoptive-transfer of allogeneic MHC-mismatched gamma/delta T-cells into lymphodepleted tumor-bearing hosts: New paradigm for the immunotherapy of breast cancer

Agency: Department of Defense, Congressionally Directed Medical Research Program

Type: Concept Award.

PI: **LOPEZ, RD**

Amount: \$75,000 direct costs, *submitted 11/2008*

Project Goal: Here, we propose to develop a mouse model to assess how allogeneic $\gamma\delta$ -T cells derived from MHC mis-matched donors can be adoptively-transferred into tumor-bearing recipients which are first lymphodepleted (transiently immunosuppressed). The underlying hypothesis of our proposal states that in the transiently lymphodepleted mouse, it will be possible for adoptively-transferred MHC-mismatched $\gamma\delta$ -T cells to provide innate antitumor activity, despite the fact that they will eventually be rejected.

CONCLUSIONS

On the basis of the work completed from this award, we now hypothesize that in the majority of tumor-bearing hosts (mice or humans), the $\gamma\delta$ -T cell compartment is irreversibly "damaged" or "exhausted" – this possibly occurring in a tumor-dependent manner, quite possibly the result of tumor cells inducing apoptosis in tumor-reactive $\gamma\delta$ -T cells. Accordingly, we propose that the use of patient-derived (i.e., **autologous**) $\gamma\delta$ -T cells for cancer immunotherapy may be undesirable or even impossible in most cases. It is in this specific background of these findings that we have been able to refine our thinking. Thus, we now put forth the highly novel concept of using adoptively transferred **allogeneic** $\gamma\delta$ -T cells for the treatment of advanced or recurrent breast cancer. Key to this concept is the presumption that in contrast to endogenous $\gamma\delta$ -T cells present in tumor-bearing hosts, $\gamma\delta$ -T cells obtained from healthy donors will be "undamaged" – and thus, in theory, will be more effective against cancer cells. In proposing this allogeneic $\gamma\delta$ -T cell therapy model, we invoke both biological as well as clinical reasons why the introduction of allogeneic (donor-derived) $\gamma\delta$ -T cells might best be undertaken in the setting of an allogeneic hematopoietic stem cell (HSC) transplant. Alternatively, this could also be accomplished after transient lympho-depletion of the tumor bearing host, allowing for the temporary introduction of donor-derived $\gamma\delta$ -T cells – without need for a full allogeneic HSC transplant. In summary, the biological principles learned from these studies will be crucial to the rational design and subsequent execution of the next generation of human $\gamma\delta$ -T cell-based immunotherapies directed against breast cancer or other human malignancies.

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APPENDIX none